SUPPLEMENTARY INFORMATION

A multivalent pseudopeptide used to target cell-surface nucleoproteins: a potential approach in cancer therapy.

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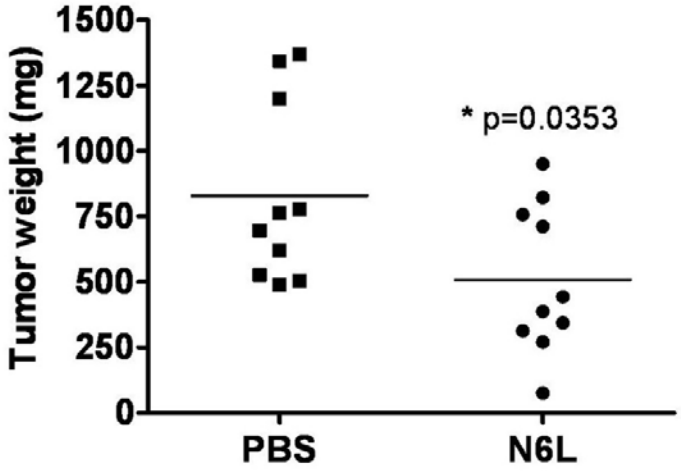
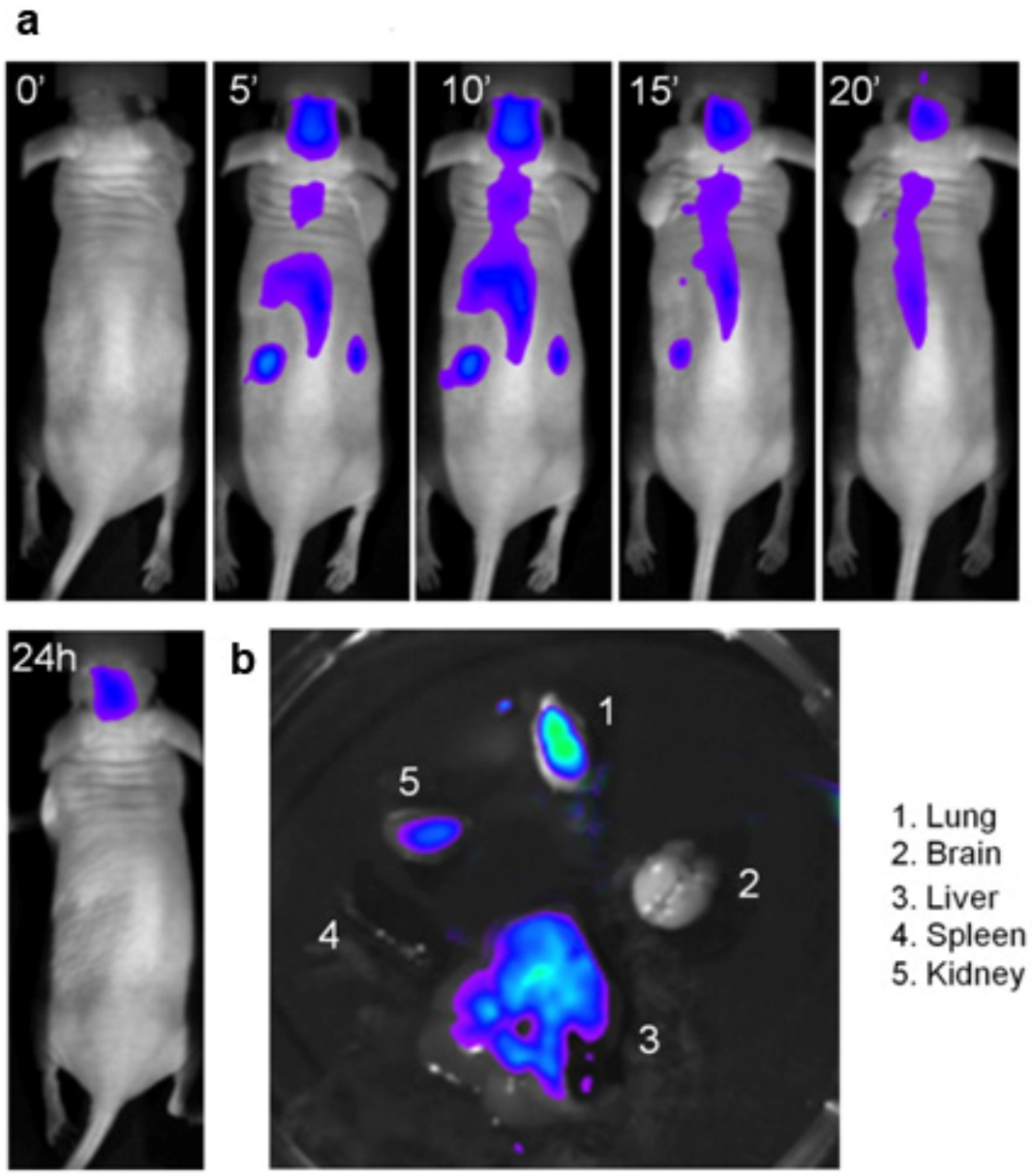
Supplementary Methods

Supplementary Fig.1



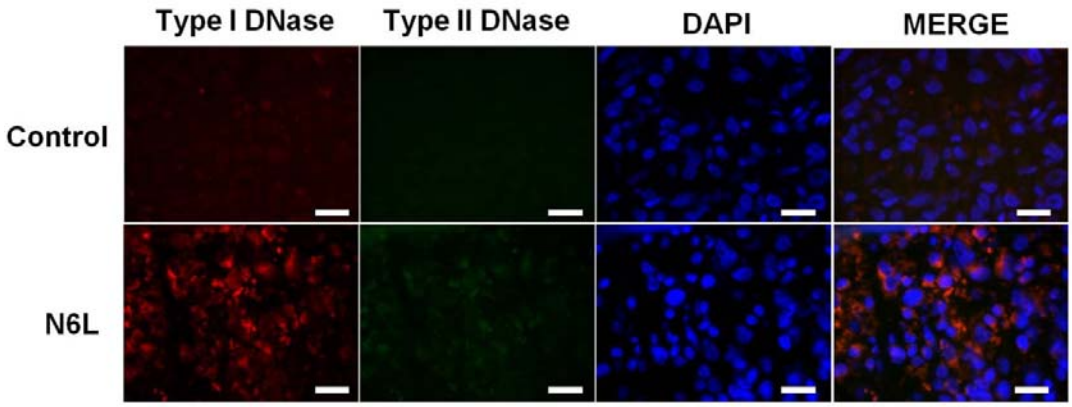
Supplementary Fig.1: N6L inhibits tumor growth of PC3 ectopic xenografts in nude mice. PC3 cells (2 x 10^6) were injected subcutaneously into the right flank of female nude mice. When tumor reached about 50 mm^3, mice were randomly separated into two groups (n = 10 per group). Mice were treated with PBS (control group) or NucAnt 6L 1 mg/kg three times per week by i.p. injection. At the end of the PC3 xenograft experiment, each tumor was excised and weighed. Data are expressed as tumor weight from each mouse. Mean tumor weight is indicated for each group. Bars, ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ are statistically significant compared to control (Student's t test).

Supplementary Fig.2



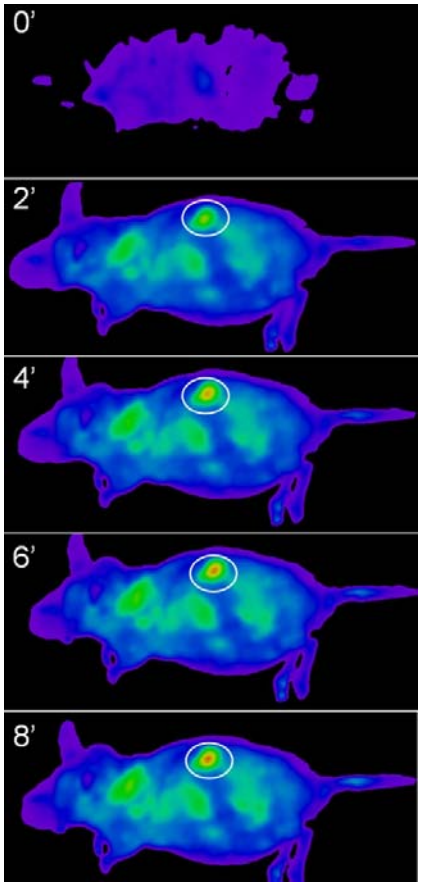
Supplementary Fig.2: IV injection of alexa 633-labeled N6L into mice without tumors. (**a**) Representative fluorescence images in mice imaged after 0 to 24 h of 10 µg alexa 633-labeled N6L injected into the tail vein. (**b**) *Ex vivo* macroscopic fluorescence images of organs extracted 24 h after injection of 10 µg alexa 633-labeled N6L.

Supplementary Fig.3



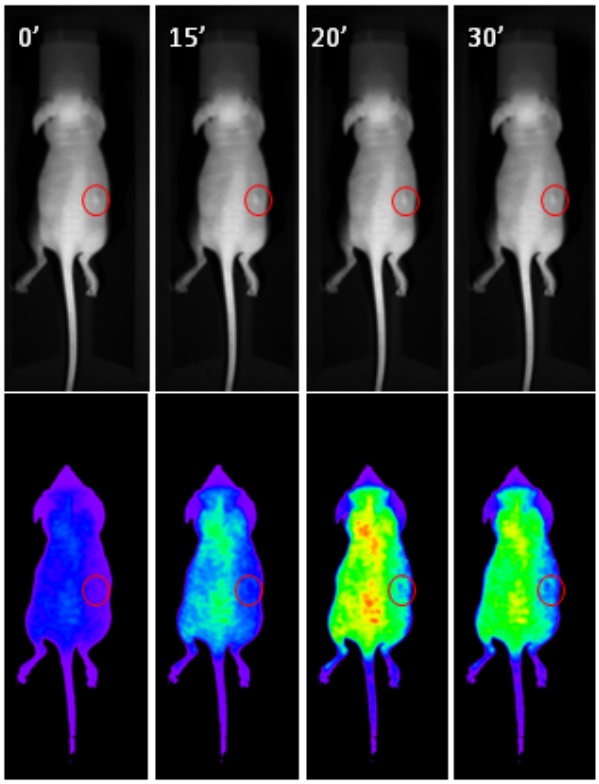
Supplementary Fig.3: N6L induces caspase-dependent apoptosis *in vivo* in PC3 tumors. PC-3 derived tumors were removed, frozen, and then stained by TUNEL assay, as described by the manufacturer. Red signal corresponds to Type I DNase activation dependent on caspase 3 activation. Green signal corresponds to Type II DNase activation independent of caspase activation. Scale Bar represents 50 µm.

Supplementary Fig.4



Supplementary Fig.4: *In vivo* imaging of N6L distribution in mice bearing U373 MG tumors. Representative fluorescence images in mice imaged after 0 to 8 min of 200 μg rhodamine-labeled N6L injected into the tail vein. Increased fluorescence was observed in tumor (red dot in white circle) 3 min after injection of rhodamine-labeled N6L, increased until 6 min, and reached a plateau at between 7 and 8 min.

Supplementary Fig.5



Supplementary Fig.5: *In vivo* imaging of N6L template distribution in mice bearing MDA-MB 231 tumors (red circle). Representative fluorescence images after injection of N6L-alexa fluor 633 template intravenous into the tail vein (0 to 30 min). A widespread distribution was observed without tumor enrichment.

Supplementary Table 1

MS/MS sample name	Protein name	Protein molecular weight (Da)	Number of unique peptides	Number of unique spectra	Peptide sequence	Previous amino acid	Next amino acid	Mascot Ion score	Mascot Identity score	Observed m/z	Actual peptide mass (AMU)	Calculated +1H Peptide Mass (AMU)	Spectrum charge	Actual minus calculated peptide mass (PPM)
W07517MS (F061886)	PREDICTED: similar to nucleolin	100,745	7	8	ALELTGLK	K	V	47.8	40.1	422.7627	843.5098	844.5146	2	3.586
			7	8	TGISDVFAK	K	N	58.1	41.0	469.2557	936.4957	937.4996	2	4.142
			7	8	TGISDVFAK	K	N	68.8	40.9	469.2569	936.4982	937.4996	2	6.876
			7	8	NDLAVVDVR	K	I	59.5	41.2	500.7717	999.5278	1,000.5428	2	-7.161
			7	8	EVFEDAAEIR	K	L	46.5	41.8	589.7893	1,177.5629	1,178.5694	2	1.068
			7	8	IVTDRETGSSK	R	G	59.7	42.0	596.8101	1,191.6046	1,192.6174	2	-4.165
			7	8	TEADAEKTFEEK	K	Q	45.7	40.1	699.3272	1,396.6388	1,397.6438	2	2.036
			7	8	VTQDELKEVFEDAAEIR	K	L	47.3	41.1	996.5096	1,991.0036	1,991.9926	2	9.456
			7	8	VTQDELKEVFEDAAEIR	K	L	57.0	40.8	664.6659	1,990.9741	1,991.9926	3	-5.374
			2	2	GPSSVEDIK	K	A	52.7	42.0	466.2402	930.4648	931.4737	2	-1.139
W07520MS (F061885)	nucleophosmin 1 isoform 1 [Homo sapiens]	32,557	2	2	MSVQPTVSLGGFEITPPVV LR	K	L	51.7	40.3	748.4142	2,242.2192	2,243.2113	3	7.037

Supplementary Table 1 Identification of N6L-interacting proteins by mass spectrometry

Supplementary Table 2

		Control	NaB	N6L		
			10 mM	5 µM	20 µM	50 µM
MDA-MB 231	Annexin V +/PI -	0.08 ± 0.28	8.30 ± 0.02	0.80 ± 0.05	3.77 ± 0.21	11.21 ± 0.16
	Annexin V +/PI +	0.43 ± 0.07	33.05 ± 0.09	2.11 ± 0.04	5.37 ± 0.29	13.80 ± 1.76
	Annexin V -/PI +	1.54 ± 0.01	1.60 ± 0.74	1.57 ± 0.05	1.81 ± 0.54	2.95 ± 1.35
MDA-MB 435	Annexin V +/PI -	1.79 ± 0.15	4.26 ± 1.01	2.25 ± 0.37	6.19 ± 3.11	12.22 ± 2.58
	Annexin V +/PI +	2.04 ± 0.03	6.89 ± 0.21	4.38 ± 0.43	18.45 ± 1.03	48.03 ± 4.72
	Annexin V -/PI +	0.81 ± 0.05	3.47 ± 0.53	2.98 ± 0.24	7.19 ± 1.91	4.87 ± 7.92

Supplementary Table 2 N6L induces apoptosis in MDA-MB 435 and 231 cells. Cells were treated or not (control) for 48 hours with various concentrations (5, 20, and 50 µM) of N6L or with 10 mM of sodium butyrate (NaB) as positive control. Cells were then stained with Annexin V-FITC and propidium iodure and analyzed by FACS. Data represent the mean percentage of cells ± standard deviation.

Supplementary Movie (See File N6L2.mp4)

Time-lapse imaging of MDA-MB 231 cells MDA-MB 231 cells were placed in an incubation chamber (37°C, 5% CO_2) equipped with a time-lapse imaging system (BioStation IM; Nikon). After adding Alexa Fluor 488 labeled Nucant6L (10 μM) and Hoechst (1 μg/ml) for nucleus staining, images were captured at 10 min intervals over 14 h, then digitized with BioStation IM viewer software.

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Supplementary Methods

Peptides constructs Synthesis of N4L, N5L, N6L, and N8L was as described for HB-19 (Callebaut et al., 1996), except that the lysine-rich template is a 3_{10} helical matrix composed respectively of 4, 5, 6, and 8 repeats of Lys-Aib-Gly, the pseudotripeptides Lysψ[CH_2N]Pro-Arg being grafted onto the ε NH_2 of the matrix Lys residues. Synthesis of the reduced peptide bond Lysψ[CH_2N]Pro can be classically formed on the resin by reductive amination of N-Boc Lys(Boc) aldehyde in dimethylformamide containing 1% acetic acid. However, it has been shown (Cushman and Ob, 1991) that the secondary amine of a proline residue reacts with the aldehyde to form an enamine with loss of chirality at the modified Lys residue in this case. To circumvent this problem, the protected dipeptide Boc-Lys(Boc)-Pro-OBzl was prepared and the amide bond selectively reduced with diborane. The resulting stereochemically pure Boc-Lys(Boc)ψ[CH_2N]Pro-OH was then introduced during the solid phase synthesis. After HF cleavage and HPLC purification, the final optically pure compounds were named N4L, N5L, N6L, and N8L. Fluorescent conjugates were prepared starting from an N6L-Cys derivative built by elongating the C-terminal sequence of the template with the Cys-Aib-Gly sequence. N6L-Cys was purified and analyzed as described above. N6L-Cys was then allowed to react, respectively with equimolar quantities of alexa fluor 488 C5-maleimide, alexa fluor 633 C5-maleimide, or rhodamine Red C2-maleimide (Thermo Fisher Scientific) in dimethylformamide. N6L-alexa fluor 633 template was prepared according to the same procedure. The various conjugates were then purified by HPLC and lyophilized.

Cell culture MOLT-4, Jurkat, A20, Raji, HL-60, HL-60 MX2, RAW, HCT116, HCT116 p53-/-, MDA MB-231, MDA MB-435, PC3, U87-MG, U373-MG, B16-Bl6, and Renca cell lines were purchased from ATCC (American Type Culture Collection). Murine lymphoma T29, which was a gift from Dr P. Kastner (IGBMC, Strasbourg) and human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (Cambrex Bio-Sciences.)

Biotinylated N6L pull-down experiments The detection of cell surface protein(s) recognized by N6L was adapted from a previously described method (26).

Cell-surface protein biotinylation Biotinylation of cell-surface proteins was carried out using the Cell Surface Protein Isolation Kit (Pierce/ Thermo Fisher Scientific) according to the manufacturer's instructions.

Western blot analysis Cells were lysed and analyzed by SDS-PAGE and Western blotting. The following primary antibodies were used: monoclonal anti-nucleolin mouse antibodies (Santa Cruz Biotechnologies), anti-nucleophosmin and anti β tubulin (Abcam), anti-GAPDH (Ambion), and polyclonal rabbit anti-nucleolin (Abcam).

Affinity isolation experiments

The detection of cell surface protein(s) recognized by N6L was adapted from a previously described method(Page et al., 2009). MDA-MB 231 or Raji cells were incubated for 1 h at room temperature in serum-free RPMI 1640 culture medium (Biomedia) containing biotin-labeled N6L. After washing the cells once in PBS containing 1 mM EDTA, nucleus-free cell extracts were prepared in lysis buffer E (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 µl/ml protease inhibitors obtained from Sigma-Aldrich, 0.5% Triton X100, 1 mM NaF, and 1 mM Na_3VO_4.) The complexes formed between biotin-labeled N6L and putative cell-surface interacting proteins were isolated by purification of the extracts, using 100 µl avidin-agarose (Pierce, Thermo Fisher Scientific) in PBS containing 1 mM EDTA, 1 mM NaF, and 1 mM Na_3VO_4. After overnight incubation at 4°C, the samples were washed extensively with the same buffer. The purified proteins were subjected to SDS-PAGE and/or Western blot analysis. Identification of the protein of interest was deduced from mass spectrometry analysis (nano LC-MS/MS) as previously described(Page et al., 2009).

Surface plasmon resonance analysis Nucleolin (Abnova, H00004691-P01) and nucleophosmin (Abnova, H00004869-P01) used in this experiment were full length recombinant protein with GST tag. No binding was detected using GST protein. All binding experiments were carried out at 25°C with a constant flow rate of 20 µl/mn, using the BIACORE 3000 system. Global analysis was carried out using the simple Langmuir binding model. Specific binding profiles were obtained after subtracting the response signal from that

of the peptide control. The fitting to each model was judged by the reduced chi-square and randomness of residue distribution.

Protein preparation for in-gel digestion

The gel pieces were successively washed with 50 µl of 25 mM NH_4HCO_3 and 50 µl of acetonitrile (three times), and dehydrated with 100 µl acetonitrile before reduction in the presence of 10 mM dithiothreitol in 25 mM NH_4HCO_3 (1h at 57°C) and alkylation in the presence of 55 mM iodoacetamide in 25 mM NH_4HCO_3. For tryptic digestion, the gel pieces were resuspended in 2 volumes of trypsin (12.5 ng/µl; Promega V5111) freshly diluted in 25 mM NH_4HCO_3 and incubated overnight at 37°C. The digested peptides were then extracted from the gel in a solution containing 34.9% H_2O, 65% acetonitrile and 0.1% HCOOH and directly analyzed by nanoLC-MS/MS.

Chromatography conditions for NanoAcquity

Analysis was carried out using a nanoACQUITY Ultra-Performance-LC (UPLC; Waters). Samples were trapped on a 20 × 0.18 mm, 5 µm Symmetry C18 precolumn (Waters), and the peptides separated on an ACQUITY UPLC® BEH130 C18 column (Waters), 75 µm×200 mm, particle size 1.7 µm. The solvent system was 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). Trapping was conducted for 3 min at 5 µl/min with 99% of solvent A and 1% of solvent B. Elution was carried out at a flow rate of 400 nl/min using 1–40% gradient (solvent B) over 35 min at 45°Ck, followed by 65% (solvent B) over 5 min.

MS and MS/MS conditions on SYNAPT mass spectrometer

MS and MS/MS analyses were carried out using a SYNAPT™ apparatus, a hybrid quadrupole orthogonal acceleration time-of-flight (TOF) tandem mass spectrometer (Waters) equipped with a Z-spray ion sourcel, and a lock mass system. Capillary voltage was set at 3.5 KV and the cone voltage at 35 V. Mass calibration of the TOF was achieved using phosphoric acid (H_3PO_4) in the [50;2000] m/z range. Online correction of this calibration was carried out with Glu-fibrino-peptide B as the lock-mass. The ion $(M+2H)^{2+}$ at m/z 785.8426 was used to calibrate MS data and the fragment ion $(M+H)^{+}$ at m/z 684.3469 was used to calibrate MS/MS data during the analysis.

For tandem MS experiments, the system was operated with automatic switching between the MS and MS/MS modes (MS 0.5 s/scan on m/z range [250;1500] and MS/MS 0.7 s/scan on m/z range [50;2000]). The three most abundant peptides (intensity threshold 60 counts/s), preferably doubly and triply charged ions, were selected in each MS spectrum for further isolation and CID fragmentation with two energies set using a collision energy profile. Fragmentation was achieved using argon as the collision gas. The complete system was fully controlled by MassLynx 4.1 (SCN 566; Waters).

Data analysis and protein identification

Raw data collected during nanoLC-MS/MS analyses were processed and converted with ProteinLynx Browser 2.3 (Waters) into .pkl peak list format. Normal background subtraction type was used for both MS and MS/MS with 5% threshold and polynomial correction of order 5, and deisotoping was carried out. MS/MS data were analyzed using the MASCOT 2. 2. 0. algorithm (Matrix Science) to search against the UniProtKB/Swiss-Prot database, version 54.8, concatenated with reverse copies of all sequences (2×349,480 entries). Spectra were searched with a mass tolerance of 15 ppm for MS and 0.07 Da for MS/MS data, allowing a maximum of one missed cleavage site by trypsin, and with carbamidomethylation of cysteine residues and oxidation of methionine residues specified as fixed and variable modifications, respectively. Protein identifications were validated when at least two peptides were found with an Mascot ion score greater than 35 for each MS/MS spectrum.

For estimation of the false-positive rate in protein identification, a target-decoy database search was conducted. Criteria used for protein identification followed the general guidelines for reporting proteomic experiments (MIAPE; http://www.psidev.info).